International Journal of Neurology Sciences

ISSN Print: 2664-6161
ISSN Online: 2664-617X
IJNS 2024; 6(1): 15-22
www.neurologyjournal.in
Received: 10-11-2023
Accepted: 15-12-2023

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Neurobehavioral, histopathological and immunohistochemical effects of *Ginkgo biloba* and *Cichorium intybus* hydro-alcoholic extracts in experimentally alzheimer rats

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DOI: https://doi.org/10.33545/26646161.2024.v6.i1a.20

Abstract
Impaired memory and cognitive function are the main features of Alzheimer's disease (AD). Unfortunately, currently available treatments cannot cure or delay AD progression. Moreover, the blood-brain barrier hampers effective delivery of treatment to the brain. Therefore, we aimed to evaluate the impact of hydroalcoholic extract of *ginko biloba* and *cicurium intybus* as protective compound from neurodegenerative effect of Alzheimer disease in rat. Forty male rats were divided into five groups (eight rats each), being: C-: control negative (received 1 ml of distilled water), C+: aluminum chloride (AlCl3) (100 mg/kg, p.v.) for 28 days (positive control), G: Ginkgo Biloba (120 mg/kg, p.o.) one hour before the induction of Alzheimer's with AlCl3 (100 mg/kg.b.w.) for 28 days, C: *Cicurium intybus* (500 mg/kg.bw p.o) one hour before the induction of Alzheimer's with AlCl3 (100 mg/kg. bw) for 28 days and G+C: combination of GB + CI one hour before Alzheimer's induction with AlCl3. The result of neurobehavioral tests revealed that drenched of 100mg/kb.bw of $Alcl_3$ significant decrease (p>0.05) the number of squares crossed by four legs /3 minutes, swimming test and Y-maze test in all treated groups as compared with control negative group. However, the tau protein overexpressed in control positive group as compared with protective groups. Histopathological slides note there were a necrosis, hemorrhage with the presence of inflammatory cells in the subiculum area of the hippocampus in brain of control positive group while the best protective effect showed in combination group. In conclusion, the combination of two antioxidant herbs is an effective, safe, and non-invasive approach with superior cognitive function capabilities compared to each one alone.

Keywords: $AlCl_3$, tau protein, *Ginko Biloba*, *Cicurium intybus* and alzheimer disease

Introduction
Alzheimer's disease (AD) is marked by a gradual deterioration in memory, attention, and other cognitive abilities. AD has a lengthy pre-symptomatic period, and it is believed that degenerative changes in the brain start many years before cognitive issues become apparent (Jack *et al*., 2010; Knopman *et al*., 2021) [7, 22]. Approximately 70 million individuals have AD in the world, characterized by cognitive deficits in memory and other cognitive functions, caused death within 3-9 years following diagnosis (Chen *et al*., 2021) [7]. Aluminum is abundant in our environment, and for a long time, substances such as clay, glass, and alum have been used in manufacturing. Aluminum, a widely recognized neurotoxin, hinders over 200 physiologically important processes and has numerous detrimental effects on humans, animals, and plants (Zghari *et al*., 2018) [43]. However, the specific molecular processes by which aluminum causes neurotoxicity are still not understood. The detrimental consequences of aluminum absorption and accumulation, such as a gradual brain disorder that ultimately leads to dementia, are widely acknowledged (Eichenbaum *et al*., 2007) [11]. Hippocampus plays a vital role in memory formation and retrieval. While traditionally believed to primarily contribute to declarative memory, current studies suggest that it may also have a role in other cognitive functions such as short-term memory, creativity, and decision-making, which are not directly associated with declarative memory (Ajeleti *et al*., 2023) [2].

Region I of the hippocampus (CA1) corresponds to a specific part of the hippocampal formation. The entorhinal cortex layer V receives input from area CA1, which is the initial region in the hippocampal circuit to generate a significant output pathway. Aluminum predominantly accumulates in the hippocampus and frontal cortex of the brain, which are recognized as particularly susceptible regions in neurodegenerative disorders (Farombi *et al*., 2019) [13]. It causes cytoskeleton proteins to misfold, which causes amyloid beta plaques and tau neurofibrillary tangles to form in the brain (Kawahara *et al*., 2001) [21]. Ginkgo biloba (GB), an acknowledged botanical therapy, has been employed in China since ancient times (Al-Ameedi *et al*., 2023) [3]. GB extract mostly contains flavonoids and terpenoids (Xie *et al*., 2022) [41]. GB may help treat AD because it contains different parts that fight free radicals, inflammation, apoptosis, and protect against mitochondrial dysfunction, amyloid formation, and A aggregation. Additionally, GB is believed to modulate ion homeostasis and phosphorylation of the tau protein (Chen *et al*., 2019) [6]. Many studies indicate that GB notably enhanced the cognitive abilities of AD in rodents' model (Ward *et al*., 2002; Tian *et al*., 2012) [40, 38]. *Cichorium intybus* (CI) was used in ancient Rome, Greece, and Egypt to enhance metabolism and digestion (Janda *et al*., 2021) [18]. Furthermore, it functions as both a vegetable and a pasture plant and includes glycosides and triterpenoids. These compounds hinder glutamatergic transmission and improve GABAergic transmission (Javed *et al*., 2020) [19]. This study aimed at investigating the protective effects of hydroalcoholic leaf extract of GB and CI on Aluminum chloride induced hippocampal toxicity using behavioral protocols (open field, swimming rank and Y-maze), histological and histochemical principle (Congo red and Hematoxylin & DAB stains) in rat model.

Materials and Methods

Chemicals

All the chemicals that utilized in this present research were of analytical quality and sourced from several firms specializing in medical and commercial services.

Plants

In September 2023, dried leaves of GB and CI were bought at a local market in the Al-Hilla province, Iraq. The leaves were carefully pulverized with a blender.

Hydro-alcoholic Extraction

The plant leaves were extracted by using hot plate apparatus with stirring, as described by Harborne (1984) [15] and Al-Ameedi and Nahi (2019) [4].

Animals

A total of 40 male albino rats, aged about 3 months and weighing around 160-200 g, were acquired from the animal house at the College of Veterinary Medicine/Al-Qasim Green University. The rats were provided with a regular pellet meal and given tap water. They were housed in specialized cages in ideal, normal settings for a period of two weeks to allow for adaptation. The ideal conditions included a 12-hour light-dark cycle and a temperature range of 20-25 °C in an air-conditioned environment. The bedding was replaced twice per week.

Experimental design

Group 1: Control group (received distilled water 1 ml)
Group 2: Aluminum chloride (100 mg/kg, p.o.) Alzheimer induction for 28 days (Chen *et al*., 2021) [7].
Group 3: Gingko biloba (120 mg/kg, p.o.) (Verma *et al*., 2020) [39], prior one hour to aluminum chloride (100 mg/kg.bw) Alzheimer induction for 28 days
Group 4: *Cichorium intybus* (500 mg/kg.bw p.o) (Noori and Mahboob, 2012) [33], prior one hour to aluminum chloride (100 mg/kg.bw) Alzheimer induction for 28 days.
Group 5: Combination of (120 mg/kg.bw GB) + (500 mg/kg.bw C.I) p.o prior one hour to aluminum chloride (100 mg/kg.bw) Alzheimer induction for 28 days.

Neurobehavioral study

1. Open felid test

This test evaluates the animal's overall locomotor activity, exploration (movement of all four legs in both forward and backward directions), rearing, and frequency of feces and urine. Placed the rats in the central area of an open field apparatus and observed their movements for 3 minutes. We recorded the number of squares traversed, instances of rearing, fecal boluses, and urine boluses. We thoroughly cleaned the arena after each attempt (Moser, 1991; Mohammed, 2000) [30]. The arena is comprised of a hardwood open box of 100 × 100 cm2. It is divided into 16 equal squares, with each little square measuring 20x20cm2, suitable for adult rats.

2. Swimming rank test

It is a glass pool with a height of 30 cm containing warmed water (30 °C). This test reflects the integration of brain function by monitoring each animal for (5-10) seconds for swimming in the pool (for adult rats' measurement was (70 x 40 x 40 cm) containing warmed water (30 °C), and the evaluation carried out by grades as following (Schapiro *et al*., 1970) [36].
Grade 0: When the nose is under the plane of water.
Grade 1: The nose with plane or above the plane water.
Grade 2: The nose and crown with, or above the plane of water while the ears are under.
Grade (3): As in grade (2) but the plane of water at the mid of ears.
Grade (4): As in grade (3) but the plane of water under the ears.

3. Y- maze test

We constructed the Y maze according to the specifications outlined by Dellu *et al*. (1992) [9]. The arm-recognition component of the Y maze has been utilized instead of object-recognition memory due to the non-essential role of the hippocampus in the latter (Mumby *et al*., 1992) [32]. The Y maze consisted of three arms that were identical in size, measuring 50 cm in length and 16 cm in width, and with sides that were 32 cm high. Each arm was fitted with two pairs of infrared photocells, positioned at distances of 21 cm and 42 cm from the ends of the arms and 4.25 cm above the floor. The arms of apparatus were numbered as A, B, and C, the A one is the beginning of the test, the rat was put in the A arm and measuring the 12 entries to different arms in which successful trail is the entry to the 3 different arms animal entrance to other arms and choose the three different trials (Deacon and Rawlins, 2006) [8].

Histopathology

The brain specimens were preserved using a 10% neutral buffered formalin solution until the histological slices were prepared. The tissues were fixed in paraffin, and several tissue slices were produced for histopathological analysis. These sections were stained using Congo red stain (Luna and Lee, 1968) [27].

Immunohistochemistry

The immunohistochemistry was performed using Dako EnVision detection immunohistochemistry kit (Envision FLEX, Dako, K8000, Denmark) and as per manufacturer's instruction.

Statistical analysis

The experimental findings were analyzed using SPSS version 16, using one- and two-way ANOVA to determine the significance of differences between control and treated adult rats. The results were presented as Mean± Standard Errors (SE), with a P-value < 0.05 indicating statistical significance. The significant levels of treatment means were tested using LSD (Joda, 2008) [20].

Results

1. Open field

Number of square crossed in arena / 3 min

The result presented in table 1 revealed that drenched of 100 mg/kb.bw of $Alcl_3$ significant decrease (p>0.05) the number of squares crossed by four legs /3 minutes in all treated groups of the study as compared with those in zero time except C- group. At the end of experiment (28 days) the combination group showed the best protective effect as compared with G and C groups respectively during experiment.

Table 1: The open field test measured the number of squares crossed by the four legs/3min

Groups N=8	Zero time Periods (Mean ± S.E)	28 days Periods (Mean ± S.E)
C+	19±1.13Aa	11±1.33Cb
C-	22±2.01Aa	19±2.04Aa
G	21±1.19Aa	15±1.94Bb
C	18±1.17Aa	13±1.67Bb
G +C	20±2.10Aa	16±2.09Ab
LSD	4.32	

Different Capital letters denote among groups statistical differences where small letters denote between periods statistical differences at (p<0.05)

Number of bolus feces /3min

Table 2 presented that there was no statistically significant difference (p>0.05) in the number of fecal boluses every 3 minutes among the groups, except in C+ group which received ALC3 without protective therapy.

Table 2: The open field test, measuring the number of boluses feces/ 3 minutes

Groups N=8	Zero time Periods (Mean ± S.E)	28 days Periods (Mean ± S.E)
C+	1.41±0.77Aa	0.45±0.94Ab
C-	1.20±0.64Aa	1.23±0.67Aa
G	1.38±0.51Aa	1.13±0.83Aa
C	1.43±0.69Aa	1.01±0.55Aa
G +C	1.39±0.77Aa	1.20±0.42Aa
LSD	0.97	

Different Capital letters denote among groups statistical differences where small letters denote between periods statistical differences at (p<0.05)

Number of urination/3min

There was no significant change in the number of urine frequency observed during the open field test among the experimental groups in all periods (p>0.05) as presented in table 3. During the experiment, the results showed a statistically significant drop (p<0.05) in the C+ group after 28 days of exposing to ALCL3 without any protective therapy.

Table 3: The open field test, measuring the number of urination/ 3 minutes

Groups N=8	Zero time Periods (Mean ± S.E)	28 days Periods (Mean ± S.E)
C+	1.33±0.82Aa	0.76±0.09Ab
C-	1.28±0.64Aa	0.93±0.10Aa
G	1.18±0.81Aa	0.83±0.07Aa
C	1.19±0.62Aa	0.71±0.55Aa
G +C	1.15±0.87Aa	1.02±0.42Aa
LSD	0.51	

Different Capital letters denote among groups statistical differences where small letters denote between periods statistical differences at (p<0.05)

2. Swimming rank test

After 28 days of the study, the results depicted in table 4 showed that there was significant decrease in swimming grade/20sec in C+, G, and C groups in comparing with C- and G+C groups. Moreover, in groups there were significant decrease in C+ and C groups as compared with C-, G and G+C groups.

Table 4: Swimming rank test (grade/20sec) of different treated groups

Groups N=8	Zero time Periods (Mean ± S.E)	28 days Periods (Mean ± S.E)
C+	4.33±0.71Aa	2.10±0.49Bb
C-	4.28±0.59Aa	3.93±0.24Aa
G	3.92±0.95Aa	3.28±0.46Ab
C	4.19±0.19Aa	2.21±0.30Bb
G +C	3.85±0.53Aa	3.72±0.91Aa
LSD	1.31	

Different Capital letters denote among groups statistical differences where small letters denote between periods statistical differences at (p<0.05)

3. Y-maze test alternate arm/3min

The results of y-maze in the different treated groups were listed in table (5) that revealed a significant decrease (p<0.05) in a all-experimental groups at 28 days. However, when comparing groups the results showed a significant decrease (p<0.05) in C+, G, and C groups as compared with C- and combination group C+G.

Table 5: The results of y-maze in the different treated groups

Groups N=8	Zero time Periods (Mean ± S.E)	28 days Periods (Mean ± S.E)
C+	2.91±0.15Aa	0.95±0.12Db
C-	3.28±0.64Aa	3.04±0.18Aa
G	3.06±0.10Aa	2.75±0.13Bb
C	3.89±0.77Aa	1.91±0.79Cb
G +C	2.05±0.90Aa	1.82±0.82Ca
LSD	0.89	

Different Capital letters denote among groups statistical differences where small letters. Denote between periods statistical differences at (p<0.05)

Taw protein score (Immunohistochemistry)

In this study, it was observed an elevation in brain tau protein in control positive group C+ compared with G, C, G+C and C- respectively as described in table 6. Moreover, the results showed a significant decrease in tau protein level in animals of combination group C+G as compared with G and C groups respectively.

Table 6: Tau protien scores in different treated groups

Group	Tau expression score	
	Mean ± SD	
C+	9**	2.12
C-	0.8	0.04
G	4.6*	1.34
C	3.6*	1.52
C+G	1.6	0.55

**: Indict a significant (p<0.05) increment in group C+ compared with groups G, CH and CH+G.
*: Indict a significant (p<0.05) increment in groups G and CH compared with the CH+G group.

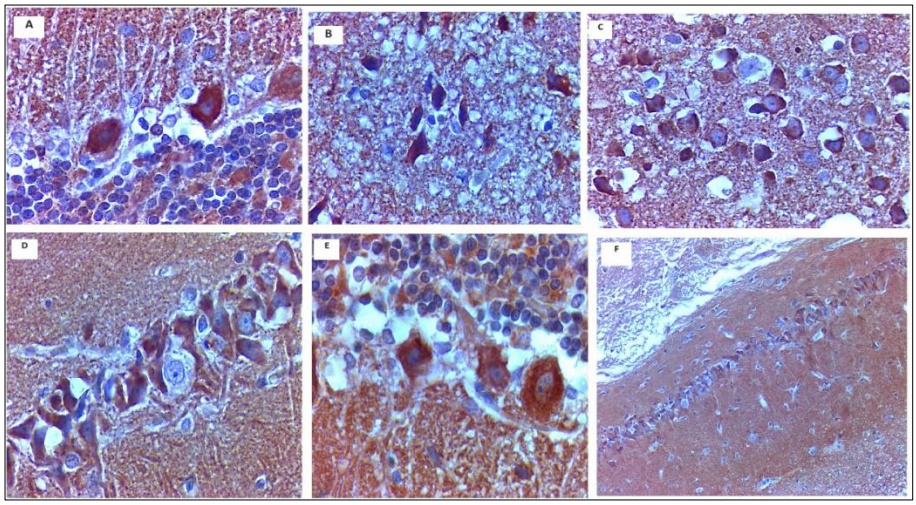

Fig 1: A/ Photomicrograph of the cerebellum of control negative group showed positive expression of Tau primary antibody in the cerebellum.100x. B/ The cerebrum of control positive group showed overexpression of Tau primary antibody in the cerebrum, where the overexpression of in neurocytes: 100x C/ The cerebrum of group G revealed the overexpression of Tau primary antibody in the cerebrum, where the overexpression of Tau was observed in neurocytes100x. D/ The hippocampus of group C. showed the expression of Tau primary antibody in the pyramidal cells in the Cornu Ammonis areas of the hippocampus 400x E/ Note positive expression of Tau primary antibody in the cerebellum, where, the positive expression of Tau was observed in Purkinje cells, however, the neurofibrillary tangles were not observed in Purkinje cells 400x. F/The hippocampus of the group CH+G note the expression of Tau primary antibody in the hippocampus, however, the expression of Tau was observed in pyramidal cells in the Cornu Ammonis area of the hippocampus 100x. (Hematoxylin & DAB stain)

Histopathological study of brain

1-C+ group: Hippocampus showed necrosis of neurocytes observed in Cornu Ammonis 1 and Cornu Ammonis 2 area forming spaces in the affected area, also the necrosis of neurocytes was observed in Cornu Ammonis 4 area. Furthermore, there was hemorrhage with the presence of inflammatory cells in the subiculum area of the hippocampus (Fig 1 A and B**).**

2-C- group: In this group, the hippocampus showed normal histological architectures (Fig 1 C).

3-G group: The slides of hippocampus showed necrosis of neurocytes in the Cornu Ammonis 4 area and subiculum area led to forming spaces in the affected area (Fig1 D).

4-C group: The cerebellum of the group C group revealed a necrosis of neurocytes in the cerebellar medulla and near the boundaries of the molecular layer and Purkinje cells (Fig1 E).

5-G+C group: The sections of this group of hippocampus showed normal histological architectures of the hippocampus (Fig1 F).

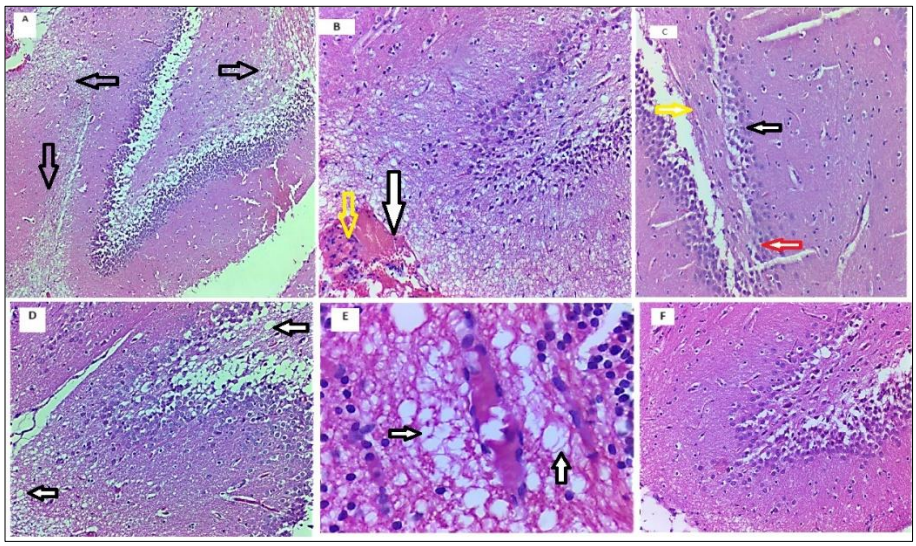

Fig 2: A/ The hippocampus of the control positive group rat showed necrosis of neurocytes (black arrow) was observed in Cornu Ammonis 1 and Cornu Ammonis 2 area forming spaces in the affected area, also the necrosis of neurocytes was observed in Cornu Ammonis 4 area. 40x. B/ Hemorrhage (white arrow) with the presence of inflammatory cells (yellow arrow) was observed in the Subiculum area of the hippocampus 100x. C/The hippocampus of the control negative group rat.Normal histological architectures of the hippocampus. Note Cornu Ammonis areas 1 and 2 (black arrow), Cornu Ammonis 4 (yellow arrow), and dentate gyrus area (red arrow). 100x. D/ The hippocampus of the group G rat. Necrosis of neurocytes was observed in the Cornu Ammonis 4 area and subiculum area led to forming spaces in the affected area. Congo red stain. 100x. E/ The cerebellum of the group CH rat. Necrosis of neurocytes (black arrow) was observed in the cerebellar medulla and near the boundaries of the molecular layer and Purkinje cells.100x. F/ The hippocampus of the group CH+G rat. Normal histological architectures of the hippocampus 40x. (Congo red stain).

Discussion

According to Ajeleti *et al*. (2023) [2] the functions of certain learning and memory have been associated with different areas of the brain such as the hippocampus and cerebellum. While the hippocampus is associated with memory of new words, faces, place and event, cerebellum has been associated with memory of learning new skills like playing an instrument etc. In our behavioral investigations, we used the AlCl3 administration to induce of Alzheimer disease as well as simulate the symptoms of depression in rats, which is similar with other reports (Liu *et al*., 2016) [26]. AlCl3 also reduced the ability of rats to learn and remember things, as demonstrated by their performance in the Y maze, a traditional test of learning and memory. These findings are in line with earlier research (Dr, 1980; Muller *et al*., 1990) [10, 31].

The findings indicated a decrease in the number of entries into and time spent in the open arm. Physical disabilities and short-term memory impairment were also considered to be contributing factors (Liu *et al*., 2014; Yan *et al*., 2016) [24]. There may be a variety of causes for the behavioral and performance deficiencies in the AlCl3- treated rats' learning and spatial memory tasks.

The main theme is that AlCl3 somehow reduces memory and learning abilities. The main question is whether this impairment is brought on by inadequate receptors in the hippocampus, declining retinal function that impairs vision, inadequate cholinergic function that reduces acetylcholine production, or a combination of these factors (Elsheikh *et al*., 2022) [12].

In contrast, in the present study the rats in G, C, and G+C groups showed a protective impact against the neurobehavioral disterbance of ALCL3 when administered prior to ALCL3. These findings consistent with many studies that concluded the powerful antioxidant abilities of *Ginko biloba* and *Cicuriun Intybus* each one alone (Rojas *et al*., 2016; Liu and Guo, 2020; Li *et al*., 2022) [35, 25, 23]. The use of all these herbs were conducive to the reduction of inflammatory processes within the nervous system (Hirata *et al*., 2019) [16].

Exposure to AlCl3 particularly through oral route with dose of 0.5 mg per body weight is toxic to brain function (Singh and Goel, 2015) [37]. This supports a hypothetical statement by Prema *et al*. (2017) [34] that aluminum exposure has neurodegenerating effect resulting in learning deficits and the documentation compiled by Frank (2006) [14] who stated that in human, aluminum inhibits learning.

The histopathological section of brain tissue revealed a clear necrosis lesion in animals of C+, G and C groups. This could be as result of increased NFTs. When the microtubule- associated protein tau clumps together to form insoluble paired helical filaments, NFTs are created inside the neuron. According to theory, as NFTs develop inside the cell, the neuron experiences stress and eventually dies. One of the main indicators of Alzheimer's disease is the existence of NFTs in nerve tissues.

Our report is in line with that of McEwen *et al*. (2016) [28]. Results also showed evidence the fading of the neuropil, few pyramidal cells that are necrotic and vascular edema as well as pyramidal cells with haphazard Nissl staining and lamina arrangement. This is an indication that despite taking preventive measures, damages were still done to the pyramidal cells post- AlCl3 treatment. The positive effects of combination of GB and CI in neurodegenerative diseases are strengthened by the findings of this investigation. The findings provided strong proof that G+C group significantly reduced the behavioral deficits in rats caused by AlCl3 treatment.

Many reports suggest that Aβ is a major pathological characteristic of AD additionally, tau protein is hyperphosphorylated in AD and accumulates in neurons. This causes abnormal mitochondrial dynamics, which, in turn, decrease the dendritic protein and dendritic spines, resulting in hippocampal-based learning and memory impairment (Ajeleti *et al*., 2023) [2]. This is because ALCL3 reduces cerebral glucose uptake, dulls brain insulin receptors, reduces PI3K-AKT signaling activity, and raises the activity of glycogen synthase kinase 3β (Adeli *et al*., 2019) [1]. These changes induce tau hyperphosphorylation. Furthermore, glucose hypometabolism initiates the process that eventually ends in Aβ aggregation (Ali and Siddique, 2019) [5]. Conversely, the overexpression of tau protien were found to be higher in the C+ group than G, C, G+C and C- respectively. These results confirm the ability of combination of G+C hydroalcoholic extract to lower Aβ and induce tau disaggregation [66]. Thus, ginkgo biloba and *Cicurium intybus* successfully reduced the pathological changes of AD.

Conclusion

To our knowledge, this study was the first evaluation of the neuroprotective effect of *Cicurium intybus* hydroalcoholic extract as alone or in combination with Ginkgo biloba. Our findings indicate that Ginkgo biloba and *Cicurium intybus* herbs are clinically significant in preventing neurodegenerative disorders with behavioral impairment, but their effectiveness in treating such diseases is limited.

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